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WO 00/75179 A1

(54) Title: CHROMATOGRAPHIC DETERMINATION OF P-GLYCOPROTEIN REACTIVE LIGANDS

(57) Abstract: A flow chromatographic system with P-glycoprotein immobilized thereto is used in a method of identifying, isolating, and characterizing ligands that are reactive to P-glycoprotein.

INTERNATIONAL SEARCH REPORT

International application No
PCT/US00/15820

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
Y,P	SPARREBOOM et al. Clinical Pharmacokinetics of Doxorubicin in combination with GF 120918, a Potent Inhibitor of MDR1-P-glycoprotein, Anti-cancer Drugs. September 1999. Vol. 10. No. 8. pages 719-728. see entire document.	4-9
Y	KUBOTA et al. Pirarubicin might partly circumvent the P-glycoprotein-mediated Drug Resistance of Human Breast Cancer Tissues. Anticancer Research March-April 1998. Vol. 18. No. 2A. pages 967-972. see entire document.	4-9

INTERNATIONAL SEARCH REPORT

International application No
PCT/US00/15820

A. CLASSIFICATION OF SUBJECT MATTER:
US CL

530/350, 355, 387.7, 388.8, 388.85, 389.7, 395, 435/7.1, 7.2, 7.23, 7.92, 7.93, 436/538, 541, 64, 161, 162, 163, 167, 173, 174, 813, 824

B. FIELDS SEARCHED
Minimum documentation searched
Classification System: U.S.

530/350, 355, 387.7, 388.8, 388.85, 389.7, 395; 435/7.1, 7.2, 7.23, 7.92, 7.93; 436/538, 541, 64, 161, 162, 163, 167, 173, 174, 813, 824

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:
STEVEN B. KELBER
PIPER MARBURY RUDNICK & WOLFE, LLP
1200 NINETEENTH STREET, N.W.
WASHINGTON, DC 20036-2412

PCT

NOTIFICATION OF TRANSMITTAL OF INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Rule 71.1)

Date of Mailing *(day/month/year)*	05 NOV 2001
Applicant's or agent's file reference 010091-144	**IMPORTANT NOTIFICATION**

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US00/15820	09 June 2000 (09.06.2000)	09 June 1999 (09.06.1999)

Applicant

GEORGETOWN UNIVERSITY

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices)(Article 39(1))(see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer Gallelle D. Gabel
Facsimile No. (703)305-3230	Telephone No. (703) 308-0196

Form PCT/IPEA/416 (July 1992)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference 010091-144	**FOR FURTHER ACTION**	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/US00/15820	International filing date *(day/month/year)* 09 June 2000 (09.06.2000)	Priority date *(day/month/year)* 09 June 1999 (09.06.1999)
International Patent Classification (IPC) or national classification and IPC IPC(7): C07K 14/00, 16/00; C12P 7/00, 29/00; G01N 33/537, 55/574, 33/577 and US Cl.: 530/350, 355, 387.7, 388.8, 388.85, 389.7,395; 435/7.1, 7.2, 7.23, 7.92, 7.93; 436/538, 541, 64, 161, 162, 163, 167, 173, 174, 813, 824		
Applicant GEORGETOWN UNIVERSITY		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 4 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 0 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of report with regard to novelty, inventive step and industrial applicability
   - IV ☒ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
09 January 2001 (09.01.2001)	20 October 2001 (20.10.2001)
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703)305-3230	Authorized officer Gailene R. Gabel Telephone No. (703) 308-0196

Form PCT/IPEA/409 (cover sheet)(July 1998)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US00/15820

I. Basis of the report

1. With regard to the **elements** of the international application:*

☒ the international application as originally filed.

☒ the description:
pages 1-31 as originally filed
pages NONE, filed with the demand
pages NONE, filed with the letter of _____.

☒ the claims:
pages 32 and 33, as originally filed
pages NONE, as amended (together with any statement) under Article 19
pages NONE, filed with the demand
pages NONE, filed with the letter of _____.

☒ the drawings:
pages 1-5, as originally filed
pages NONE, filed with the demand
pages NONE, filed with the letter of _____.

☐ the sequence listing part of the description:
pages NONE, as originally filed
pages NONE, filed with the demand
pages NONE, filed with the letter of _____.

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language _____ which is:

☐ the language of a translation furnished for the purposes of international search (under Rule23.1(b)).

☐ the language of publication of the international application (under Rule 48.3(b)).

☐ the language of the translation furnished for the purposes of international preliminary examination(under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

☐ contained in the international application in printed form.

☐ filed together with the international application in computer readable form.

☐ furnished subsequently to this Authority in written form.

☐ furnished subsequently to this Authority in computer readable form.

☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. ☐ The amendments have resulted in the cancellation of:

☐ the description, pages NONE

☐ the claims, Nos. NONE

☐ the drawings, sheets/~~fig~~ NONE

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).**

** Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).*

*** Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.*

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US00/15820

IV. Lack of unity of invention

1. In response to the invitation to restrict or pay additional fees the applicant has:

☐ restricted the claims.

☐ paid additional fees.

☐ paid additional fees under protest.

☐ neither restricted nor paid additional fees.

2. ☐ This Authority found that the requirement of unity of invention is not complied with and chose, according to Rule 68.1, not to invite the applicant to restrict or pay additional fees.

3. This Authority considers that the requirement of unity of invention is accordance with Rules 13.1, 13.2 and 13.3 is

☒ complied with.

☐ not complied with for the following reasons:

4. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this report:

☐ all parts.

☐ the parts relating to claims Nos. ____

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US00/15820

V. Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. STATEMENT

Novelty (N)	Claims 1-10	YES
	Claims NONE	NO
Inventive Step (IS)	Claims NONE	YES
	Claims 1-10	NO
Industrial Applicability (IA)	Claims 1-10	YES
	Claims NONE	NO

2. CITATIONS AND EXPLANATIONS

Claims 1-10 lack an inventive step under PCT Article 33(3) as being obvious over Ling et al. (US 4,837,306) in view of Kubota et al. (Anticancer Research, 1998) and Gianni et al. (Journal of Clinical Oncology, 1997).

Ling et al. disclose that P-glycoprotein surface antigen correlates well with multidrug resistance and therefore raises the possibility that P-glycoprotein may serve as a diagnostic marker for the detection of multidrug-resistant cells in cancer patients (see Abstract). Ling et al. specifically disclose immobilizing P-glycoprotein on artificial membrane support (nitrocellulose filters) to detect ligands (antibodies) under study for identification, isolation or characterization (see column 7, specially lines 28-32).

Ling et al. differ in failing to incorporate P-glycoprotein into a liquid chromatography system for use in order to identify the activity of certain ligands with P-glycoprotein.

Kubota et al. teach assessing the sensitivity and expression of P glycoprotein with ligands such as anthracyclines (Doxorubin and Tetrahydropyranyl Adriamycin (see page 969).

Gianni et al. teach assessing the pharmacokinetic activity of doxorubin and other ligands using high pressure liquid chromatography (see Abstract).

One of ordinary skill in the art at the time of the instant invention would have reasonable expectation of success in incorporating the teaching of Gianni in using HPLC to assess pharmacokinetic acitivity of ligands that interact with PGP such as anthracyclines as taught by Kubota, into the method of Ling which specifically disclosed immobilizing PGP onto a membrane support to detect and assess chatacterization of ligands that interact with PGP because incorporation of bioaffinity matrices such as suggested by Ling into chromatographic systems is conventional and well within ordinary skill in the art.

Claims 1-10 have industrial applicability as defined by PCT Article 33(4) in the field of combinatorial chemistry and drug screening assays.

---------- NEW CITATIONS ----------

INTERNATIONAL SEARCH REPORT

International application No
PCT/US00/15820

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C07K 14/00, 16/00; C12P 7/00, 29/00; G01N 33/537, 33/574, 33/577

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : Please See Extra Sheet.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE,EMBASE,SCISEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
Y	US 4,837,306 A (LING et al.) 06 June 1989 (06.06.89), see entire document.	1-3,10
X,P	ZHANG et al. "Development of an Immobilized P-glycoprotein Stationary Phase for on-line Liquid Chromatographic determination of Binding Affinities," Journal of Chromatography. B, Biomedical Sciences and Applications. February 2000. Vol. 739. No. 1. pages 33-37, see entire document.	1-10
Y	GIANNI et al. "Human Pharmacokinetic Characterization and In-vitro study of the Interaction between Doxorubicin and Paclitaxel in patient with Breast Cancer," Journal of Clinical Oncology. May 1997. Vol. 15. No. 5. pages 1906-1915, see entire document.	4-9

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:	
"A" document defining the general state of the art which is not considered to be of particular relevance	
"E" earlier document published on or after the international filing date	
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention	
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone	
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art	
"&" document member of the same patent family	

Date of the actual completion of the international search

22 AUGUST 2000

Date of mailing of the international search report

03 OCT 2000

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CHROMATOGRAPHIC DETERMINATION OF P-GLYCOPROTEIN REACTIVE LIGANDS

Field of the Invention:

This invention relates to immobilization of transporters on a support in a liquid chromatographic system.

Background of the Invention:

The combinatorial synthesis of chemical libraries has created an enormous pool of possible new drug candidates. Indeed, synthetic capabilities have outstripped the ability to determine corresponding biological activity. An initial step in the resolution of this problem has been the development of microliter plates which contain immobilized receptors/ antibodies. The use of these plates can rapidly reduce the number of possible candidates in a combinatorial pool from thousands to hundreds. However, assignment of relative activity within the reduced pool of compounds remains a slow and repetitive process.

The relationship between basic pharmacological processes and liquid chromatographic (LC) studies have been emphasized by the inclusion of biomolecules as active components of chromatographic systems. A wide variety of immobilized biopolymer-based LC stationary phases (BP-SPs) have been developed using proteins, enzymes, cellulose and amylose, macrocyclic antibodies and liposomes. Indeed, it has been demonstrated that the chromatographic retention and selectivity of BP-SPs are related to the properties of the non-immobilized biopolymer. For example, retention of a compound on an SP column containing immobilized human serum albumin has been used to evaluate the binding properties of the compounds to proteins.

P-glycoprotein (PGP) is a 170 to 180 kDA membrane transporter that acts as an ATP-driven drug efflux pump. The over-expression of PGP has been associated with multidrug resistance (MDR) in tumor cells and the MDR phenotype is a key factor in the failure of chemotherapeutic treatment of breast cancer. The MDR1 genes encode the PGP that is involved in the MDR phenotype. A breast tumor is two times more likely to express MDR1/PGP if it has been exposed to cytotoxic chemotherapy and almost three times more likely to be resistant to adriamycin *in vitro* if it expresses MDR1/PGP.

One approach to the development of therapeutic protocols to overcome MDR in breast cancer patients has concentrated on the inhibition of the PGP-mediated pump. For example, *in vitro* studies have demonstrated that the presence of verapamil in the incubation media increased the cytotoxicity of vinca alkaloids and anthracyline derivatives in MDR1/PGP tumor cell lines. However, a clinical trial combining verapamil and the vinca alkaloid VP16 and the anthracycline derivative adriamycin was not successful due to the cardiotoxicity of verapamil. Thus, the development of novel agents to reverse MDR1/PGP-mediated drug resistance remains a key objective in breast cancer research.

The functions of PGP have been studied using a variety of experimental formats, including detergent solutions, proteo-liposomes, membrane vesicles and native membranes. Only a few of these studies have concentrated on evaluation of ligand-binding parameters and the screening of pools of drug candidates for their PGP binding affinities presents a formidable task.

Summary of the Invention:

The invention provides for liquid chromatographic stationary phases to which are immobilized transporters, especially P-glycoproteins. These stationary phases are useful for identifying compounds by a continuous liquid

chromatographic process which provides for the characterization, isolation and/or identification of compounds that specifically bind to the immobilized transporter. Thereby, the invention provides continuous on-line processes for identifying and isolating agents that bind to transporters, especially p-glycoproteins.

Accordingly, the present invention provides substrate systems and methods for continuous on-line evaluation, identification and isolation of compounds which bind to PGP.

Description of the Invention:

It is the purpose of this invention to provide means for immobilization of membrane transporters in order to study interactions of compounds therewith. The fundamental processes of drug action, absorption, distribution and receptor activation, are dynamic in nature and have much in common with the basic mechanisms involved in chromatographic distribution. Indeed, the same basic intermolecular interactions (hydrophobic, electrostatic and hydrogen bonding) determine the behavior of chemical compounds in both biological and chromatographic environments. These properties are clearly illustrated herein using a PGP-based stationary phase (SP) system.

Although receptors and transporters play an important role in drug activity and are key targets in combinatorial screens, they have not been included in LC systems.

The use of receptors on supports comprising the steps of:

(a) immobilizing receptors on artificial membrane supports in a column,

(b) exposing the supports with the receptors to test agents at varying concentrations in a liquid chromatographic system,

(c) eluting the test agent from the column, and

(d) evaluating the elution profile of the test materials from the column has previously been disclosed. Using this method, it was possible to evaluate the interaction of the test agent with the receptor. Following elution, it is possible to directly determine molecular structure by passing the elute through other testing devices such as a mass spectrometer.

The present invention is exemplified herein using a PGP-based LC stationary phase for the study of drug-PGP interactions. The method was assessed in studies of the binding affinity of vinblastine, verapamil and cyclosporin A on PGP. Based thereon, it is anticipated that the present invention should be suitable for study of other ligand interactions with PGP in a dynamic manner.

The PGP-based stationary phase can be prepared by embedding PGP in a phospholipid monolayer of an immobilized artificial membrane (AIM) HPLC stationary phase, creating the PGP-IAM. In a second approach, PGP can be reconstituted into the phospholipid layer of liposomes that are immobilized on Superdex 200 gel beads by using freeze-thawing methods (PGPLIP). The latter approach was originally developed for the immobilization of liposomes or liposomes containing human red cell glucose transporter in chromatographic stationary phase. (See Wallsten, et al., *Biochim. Biophys. Acta* **982** (1987) 47 or Yang, et al, *Anal. Biochem.* **218** (1994) 210.)

The PGP-IAM was used to determine the PGP binding affinities of the vinblastine, cyclosporin A and verapamil. The binding affinities were assessed using frontal chromatographic techniques. The rank order of the calculated K_d values, i.e., highest affinity to lowest affinity, are consistent with previously reported values. The PGP-IAM was stable, reproducible and deemed a useful

addition to the art relating to G-ligand interactions and provides means for the use of rapid on-line screening of new agents for the treatment of MDR1/PGP resistant tumors.

Brief Description of the Figures:

Figure 1 shows elution profile of [^{3}H]VB[1nm] in frontal chromatography based on the PGP IAM column (0.5 X 0.8 cm) in the absence and in the presence of doxorubicin (200 nm) (profile B) in the mobile phase. Tris -Hcl buffer [50 mm, pH 7.4], flow rate 0.4 ml/min.

Figure 2 is a frontal analysis of interaction of PGP with verapamil on an immobilized PGP-IAM column (0.5 X 0.8 cm). The elution profiles of 1.0 nM [^{3}H] verapamil in solution with 10, 40, 60, 200 and 400μM non-radioactive verapamil (from right to left). Running buffer is 50 mM Tris-HCl, pH 7.4 at a flow rate of 0.5 ml/min.

Figure 3 is a nonlinear regression analysis of verapamil interaction with PGP-IAM. The data is from frontal analysis measurements.

Figure 4 shows zonal affinity chromatographic profiles of 100 μl of 23.5 nM [^{3}H] verapamil at a flow rate of 0.5 ml/min with 50 mM Tris-HCl, pH 7.4 buffer, (1) was from PGP-negative-IAM column, (2) was from IAM particles column, and (3) was from PGP-IAM column.

Figure 5 is a frontal affinity analysis of 1.0 nM [^{3}H] cyclosporine A was in the sample alone, (B) 50 nM cold vinblastine was supplemented in the sample, (C) 3 mM ATP was in the sample and running buffer, (D) 100 nM cold vinblastine was added in the sample. The running buffer was 50 mM Tris-NCl, pH 7.4.

Figure 6 is a frontal affinity chromatographic analysis of 1 nM [^{3}H] vinblastine with PGP-IAM on a column of 0.5 X 0.8 cm at a flow rate of 0.5

ml/min (A). It was obtained with 1.0 nM [^{3}H] vinblastine only (B). 1.0 nM [^{3}H] vinblastine supplemented with 3 mM ATP. The running buffer for both (A) and (B) was 50 mM Tris-IICl, pH 7.4 with 1.6% ethanol.

EXAMPLE 1

Materials and methods:

The following materials and methods were used in Example 1.

Preparation of immobilized P-glycoprotein based-HPLC stationary phases.

Immobilized P-glycoprotein (PGP) Artificial Membrane (IAM)

The cultured cells MDA436/LCC6MDR1 (20 x 106 cells) were harvested in 30 ml of PBS saline and homogenized for 20 sec with a Brinkmann Polytron homogenizer. The homogenates were centrifuged at 35,000 x g for 10 min and the pellets were suspended in 4 ml solubilization solution (50 mM Tris-HCl, pH 7.4, 250 mM NaCl, 0.5% CHAPS, 2 mM DTT, 5% glycerol) and stirred for 1 hr at 0° C.

Two hundred (200) mg of dried IAM particles was suspended in 4 ml receptor-detergent solution and stirred for 1 hour at 4 C. The mixture was dialyzed against dialysis buffer (150 mM NaCl, 10 mM Tris-HCl buffer, pH 7.4, 1 mM EDTA) for 72 hours at 4° C. The obtained PGP-IAM particles were washed with the buffer by centrifugation and packed in a glass column (id 0.5 cm).

Reconstitution and immobilization of PGP on Superdex 200 gel beads

The cultured cells MDA436/LCC6MDR1 (20 x 10^6 cells) were harvested in 20 ml of PBS saline and homogenized for 20 sec with a Brinkmann Polytron homogenizer. The homogenates were centrifuged at 35,000 x g for 10 min and the supernatant was discarded. The pellets were suspended in 4 ml solubilization solution [50 mM Tris-HCl, pH 7.5 containing 1.4% octyl -D-glucopyranoside,

20% glycerol, 1 mM Dithiothreitiol, 1 mM benzamidine and 0.4% phospholipid: *E.coli* bulk phospholipid: PC:PS:Cholesterol (60:17.5:10:12.5)] by stirring at 0° C for 40 minutes. Nonsoluble material was removed by centrifugation. The supernatant was applied on to a Sephadex G50 column which equilibrated with elution buffer (150 mM NaCl, 10 mM TrisHCl, 1 mM EDTA, 1 mM Benzamidine). The liposome fractions were collected and concentrated to 1 ml. The concentrated liposome solution was mixed with 50 mg dried Superdex 200 and kept in room temperature for 2 hours. The mixture of liposome and Superdex 200 was frozen at -75 C for 10 min and then thawed at 25 C for 10 min and the freeze-thaw cycle was repeated. The non-immobilized liposomes were removed by centrifugation and the resulting PGP-Superdex gel beads were packed in a LC column.

Chromatographic analysis of binding affinity of 3H VBL at PGP

The PGP-IAM column or PGP-Superdex 200 column was washed with buffer (150 mM NaCl, 10 mM Tris-HCl buffer, pH 7.4, 1 mM EDTA). The column was placed in a standard HPLC system. 10-40 ml of 3H VBL with different concentrations in buffer or 1 nM 3H-VBL plus 200 nM ADR in buffer were applied onto the columns at 0.4 ml/min. An on-line flow scintillation detector monitored the elution profile.

Binchoninic Acid (BCA) Protein Assay

The PGP-IAM and PGP-Superdex 200 packing materials were collected and the supernatants were removed. The samples were diluted with 0.1 N of NAOH to 2 ml. A protein standard (0.2-25 g protein in 50 l) was prepared with Albumin standard (Pierce) and 20 ml of reagent A mixed with 0.4 ml of reagent B. The standards and samples (50 l each) were added to triplicate wells in a plate. 200 l of BCA reagents (A+B) (Pierce) was added in each well. The plate

was incubated for 2h at room temperature and read in a spectrophotometer at 570 nm using the Softmax program for calculation of the protein amount.

Results:

The protein assay showed that for one milliliter of bed volume, about 170 mg proteins were immobilized on IAM column and about 10 mg proteins were immobilized on Superdex 200 column. The chromatographic results obtained with PGP-IAM column or PGP-Superdex 200 column indicated that the binding activity of PGP was retained after immobilization. For example, 3H-VBL was retarded on a PGP-IAM column (0.5 x 0.8 cm) and the retention volume was 13.3 ml at the concentration of 1 nM (profile A in Fig 6) at flow rate: 0.4 ml/min. When a displacer ligand, ADR (200 nM), was included in the mobile phase, the retention volume of 1 nM [3H]-VBL was decreased from 13.3 ml to 6.5 ml (profile B in Fig 1). This indicated that the retardation was partially due to the specific binding to saturable binding sites of PGP.

Calculation of Kd value for VBL:

The retention volumes of 3H-VBL at the different concentration in frontal chromatography were used to calculate the Kd value. The obtained Kd value for 3H-VBL determined in this technique is 19 ± 20 nM; that is consistent with the reported value, 36 ± 55 nM. These results indicated that PGP-based chromatographic stationary phase can be used for the evaluation of PGP-substrate interactions.

Calculation of the binding capacity of the PGP-SP:

Approximately 24 mg of protein was immobilized on 100 mg of IAM support (0.24 mg protein/mg IAM) and the support was packed into a 0.8 cm x 0.5 cm ID column {volume 0.2 ml). When 1 nM VBL was pumped through the column, the retention volume was 13.3 ml indicating that the column

retained 13.3 pmoles of the substrate before saturation or 0.133 pmoles VBL per mg PGP-SP. Since neither the PGP-SP nor the chromatographic conditions used in these experiments were optimized, the results indicate that a PGP-SP prepared and operated under optimum conditions will have sufficient capacity for screening of complex mixtures for PGP substrates.

EXAMPLE 2:

On-line screening of complex mixtures for PGP substrates

Step 1: Initial Chromatographic Screen:

The Initial Chromatographic Screen has been described above.

Step 2: Secondary Chromatographic Screen:

The primary fractions isolated in the Initial Chromatographic Screen and screened are concentrated under reduced pressure to a volume of approximately 0.5 ml and then introduced onto the PGP-SP LC/MS system described above showing the PGP-SP LC/MS system used as the Secondary Chromatographic Screen.

Chromatographic Step A:

The primary fraction is injected on the PGP-SP column and the switching valve is be set to direct the flow from the PGP-SP column to the storage container. This configuration of the system is maintained until the elution volume {or k' } of the least retained marker PGP-substrate {i.e., VBL, TAX, or ADR} has been reached. Since the previous studies have established the relationship between k' and Kd, this procedure directs all of the compounds in the primary fraction with a Kd less than that of the lowest marker PGP-substrate to the SC. These compounds will not be lost and will be available for subsequent analysis.

Chromatographic Step B:

After the k' of the least retained marker PGP-substrate has been reached, the switching valve is rotated and the flow from the PGP-SP column is directed onto the C18 column for the analytical separation of the compounds eluted from the PGP-SP. Since the mobile phases employed on the PGP-SP primarily consists of aqueous buffers it is assumed that the compounds will not elute from the C18 under these conditions and will concentrate at the top of the analytical column. Once the compounds have been washed off the PGP-SP {the initial cut-off is set at low-column volumes after the k' of the highest retained marker PGP-substrate}, the switching valve is rotated to its initial position allowing for a recharging of the PGP-SP) and a second HPLC pump is automatically brought on-line to the C18 column and a gradient elution program begun.

Chromatographic Step C:

The gradient elution program elutes the compounds from the C18 column and into the pre-MSD splitting valve. A portion of the compounds eluted from the C18 column is directed through the splitter to collection tubes placed in an automatic fraction collector and stored for use. The remaining portion of the compound is directed to the HP 1100 MSD and the corresponding mass spectrum obtained. The automatic fraction collector is controlled by a signal from the MSD. When the slope of the total ion current detected by the MSD changes in a positive direction, the automatic fraction collector moves from waste to the next available collection tube and when the ion current returns to a preset level, the automatic fraction collector returns to waste.

Step 3: Additional Chromatographic Screens

Once the fractions collected from the Secondary Chromatographic Screen have been assayed, the active fractions can be chromatographed again on another

PGP-SP LC/MS system. In this system the C18-SP is be replaced by a cyanopropyl-SP. The use of a second SP with different molecular interactions will increase the possibility that peaks that co-eluted on the C18-SP will be separated in the next screen.

Alternative approaches include other chromatographic phases such as the aminopropyl or phenyl bonded SPs. In addition, an immobilized HSA-SP can also be employed in order to eliminate compounds that display extensive protein binding and, therefore, limited bioavailability. The fractions can be collected as described above and assayed in accord with the teachings herein.

Steps 1 and 2 - Throughput:

Once in operation, it is expected that the Initial Chromatographic Screen should take approximately four hours per extract and the Secondary Chromatographic Screen of the primary fractions an additional 4 hours for a total of eight hours and three extracts could be processed per day. This assumes that only one Initial Chromatographic System is utilized and the systems are run sequentially. If a second Initial Chromatographic System is brought on-line, the throughput should be about 6 extracts/day and 1000 extracts/year. Of course, these throughput conditions are exemplary and can be varied by one skilled in the art, as desired.

EXAMPLE 3:

Preparation of mg or greater quantities of active lead compounds:

A flow-chart for the isolation of the active compounds can be developed from the chromatographic experiments. The biological extracts containing these compounds can be processed using a flow-chart. For illustrative purposes, a sample flow chart is presented:

A. Initial Chromatographic Screen: Take the 3rd fraction after the VBL retention volume.

B. Secondary Chromatographic Screen: Inject the fraction obtained from step A and collect the fraction eluting at 25 minutes.

C. Third Chromatographic Step: Inject the fraction obtained from Step B on the chromatographic system and collect the fraction eluting at 20 minutes.

D. Fourth Chromatographic Step, etc., until homogeneity is obtained.

If required, multiple columns can be used is the Initial Chromatographic Screen and larger format columns, i.e., a standard 150 mm x 4.1 mm ID column or greater, can be employed in the Second Chromatographic Screen. Since the initial process has the capacity to process kg quantities of botanical extracts, mg and greater quantities of active lead candidates can be prepared. Thus, enough compound can be isolated to fully characterize the molecular structure of an active lead compound and to fully characterize the *in vitro* activity of this compound. If the compound proves promising, larger quantities could also be produced for preliminary *in vivo* studies if an adequate chemical synthesis has not been developed using methods of chromatographic isolation of the active compounds from complex biological matrices known in the art.

In view of the above the PGP-SP can be used to characterize ligand-biopolymer interactions, including the direct determination of binding affinities; the identification of specific sites at which a ligand binds; elucidation of ligand-ligand binding interactions including competitive and allosteric interactions.

Using the method described herein, it is possible to undertake studies with lead drug candidates isolated in the screening program and known PGP substrates in order to identify PGP binding sites and ligand-ligand binding

interactions and to create quantitative structure-binding relationships for directed combinatorial synthesis.

EXAMPLE 4:

Determination of PGP-substrate - Drug Candidate Binding Interactions on PGP

a. **Experimental Approach:**

Both frontal and zonal LC techniques can be used in these studies. In general, the technique followed is outlined as: 1) The marker PGP-substrates [3H]-VBL, [14C]-ADR and [3H]-TAX is be used as the "solutes" and they are injected individually onto the PGP-SP column and their k's measured; 2) the drug candidates isolated and identified in the chromatographic screening process are the "displacers". Each "displacer" is added individually to the mobile phase and its concentration is systematically increased during a series of experiments; 3) the effect of the "displacer" concentrations on the k's of the "solutes" can then be measured and the Kd's of VBL, ADR and TAX established as well as the independence or overlap of their respective binding sites. Indeed, initial PGP-SP studies of the Kd of VBL have determined a Kd of 19±20 nM.

b. **Analysis of results:**

When the "solute" is chromato-graphed using a mobile phase that does not contain the "displacer", k' is directly proportional to its binding affinity to PGP. When the "displacer" is added to the mobile phase, the magnitude and direction of the resulting changes in k' can be used to determine if the "displacer" binds at the same site(s) as the marker PGP-substrates and to indicate if co-operative {k' increases}, anti-co-operative {k' decreases} and non-co-operative {k' decrease} interactions occur between the "solute" and "displacer". The relationship

between the k' of the solute and the mobile phase concentration of the displacer is expressed by the equation 1:

$$\frac{1}{(k'-x)} = \frac{VMK2[D]}{K3mL} + \frac{VM}{K3mL} \qquad \text{Eqn 1}$$

Where: VM = void volume of the column; K2 and K3 = equilibrium constants for the binding of the displacer and solute, respectively; mL = moles of the solute bound to the stationary phase; [D] = concentration of the displacer in the mobile phase; X = residual k' resulting from binding at sites unaffected by the displacer.

The term X is a constant that represents the portion of k' resulting from the binding of the solute to sites at which the displacer does not compete. If both the solute and displacer bind at only one identical site on PGP, then X = 0. Eqn. 1 predicts that when X = 0 a plot of 1/ (kl) versus [D] will produce a linear relationship with a slope of (VMK2/K3mL) and an intercept of (VM/K3mL) . The value of K2, the binding affinity constant for the displacer, can be determined directly by calculating the ratio of the slope to intercept for this plot. The inverse of the slope gives mL/Vm, which is the effective concentration of the binding sites in the column.

EXAMPLE 5:

Development of Quantitative Structure-Retention Relationships {QSRR}

a. Approach:

The development of the QSRR analysis of the data set can be run according to the procedures previously published. In brief, the lead PGP-drugs can be chromatographed on the PGP-SP under the same experimental conditions and their k's determined. The molecular structures of the compounds will also

be constructed using InsiteII 95.0 running on a Silicon Graphics Indy workstation and the molecular descriptors of the structures calculated {i.e., hydrophobicity, molecular volume, electronic distribution, molecular geometries, etc.} using Tsar V2.41, Mopac V6 and InsiteII 95.0 software and the Connolly surfaces will be calculated using MOLCAD {Tripos} software all running on the same workstation.

b. Analysis of data:

The k' s of the lead PGP-drugs can be correlated to their molecular descriptors using the multivariate regression analysis program in Tsar V2.41. The simplest possible relationship between the descriptors can be established and the predictive power of the model determined by "leave-one-out" cross-validation.

c. Pharmacophore Modeling:

The pharmacophores can be developed as previously described in the art. In brief, pharmacophores can be built using Apex-3D 95.0 software run on the Silicon Graphic Indy workstation. The pharmacophores can be constructed using all of the compounds in the set with a match superimposition greater than 0.7. 3D-QSAR equations can be derived with the site radius initially set at 1.3, the occupancy at 5, the sensitivity at 2.5 and the randomization at 500.

The data from the chromatographic, QSAR and pharmacophore modeling can provide direction to the combinatorial synthesis of new compounds to bring effective therapeutic agents to clinical trial. In addition, the knowledge gained using methods of the invention can be used to produce additional stationary phases with different biochemical targets such as Protein Kinase C. Thus, the screening of extracts and combinatorial pools can be expanded to other therapeutic areas.

Using methods of the invention, the supports with many different moieties that bind to ligands may be exposed to drugs or inhibitors, then to drugs followed by chromatographic evaluation of the presence of the drug by chromatographic means to determine whether the drug is present on the support. Using means of the invention, it is also possible to determine whether proposed inhibitors of interaction will, in fact, prevent an interaction by exposing the prepared support having the appropriate protein bound thereto to proposed inhibitors, then to the toxin or drug followed by chromatographic evaluation of the support to determine whether the toxin or drug has been prevented from binding by the inhibitor under consideration.

While the invention has been exemplified using PCP, any ligand system may be used. Supports such as hydrophilic verticle support systems may be used in the methods of the invention.

An alternative experimental approach to the determination of binding affinities is affinity chromatography. We have previously reported the synthesis of a liquid chromatographic stationary phase containing immobilized PGP and its use in the determination of PGP binding affinities.

The following examples provide further characterization of the PGP-stationary phase and disclose the use of frontal and zonal chromatographic techniques to investigate the binding of vinblastine, doxorubicin, verapamil and cyclosporin A to the immobilized PGP. The compounds were added individually to the chromatographic system with or without ATP on the running buffer. The compounds were also added in pairs using standard competitive chromatography procedures. The results of the study demonstrate that both competitive and allosteric interactions occurred during the chromatographic studies and indicate that the immobilized PGP retained its conformational mobility.

The following materials and methods were used in these additional Examples.

MATERIALS AND METHODS

Materials: Immobilized Artificial Membrane (IAM). PC particles were obtained from Regis Chemical Co (Morton Grove, IL, USA). Glass column (HR5/5) was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). [^{3}H] vinblastine and [^{3}H]cyclosporine A were purchased from Amersham Life Science Products (Boston, MA, USA). [^{3}H]verapamil was from NENTM Life Science Products, Inc (Boston, MA, USA). Vinblastine, verapamil, doxorubicin, cyclosporin, CHAPS, glycerol, benzamidine, albumin bovine, were from Sigma Chemical Co. (St. Louis, MO, USA). GF/C glass microfiber filters were from Whatman. Scintillation liquid (Flo-Scint V) was purchased from Packard Instruments (Meriden, CT, USA).

Preparation of membranes: As previously described, the PGP-positive MDA435/LCC6^{MDR1} cell line was obtained by transduction of PGP-negative expressing MDA435/LCC6 human breast cancer cells with a retroviral vector carrying MDR1 cDNA {PGP} (30). In these studies, about 80 x 10^6 cells were harvested in 10 ml of buffer A (50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 2 μM Leupeptin, 2 μM phenylmethanesulfonyl fluoride and 4 μM pepstatin). The suspension of cells was homogenized for 2 x 30 s (with a cooling period inbetween) with a Brinkmann Polytron homogenizer. The homogenized membrane was centrifuged first at 1,000 x g for 10 min, the pellets were discarded and the supernatant was collected and centrifuged at 150,000 x g for 30 min again. The membrane pellets were collected.

Immobilization of PGP on IAM particles: The membrane pellets were resuspended in 6 ml solubilization solution (50 mM Tris-HCl, pH 7,4, 500 mM

NaCl, 15 mM CHAPS, 2 mM DTT, 10 % glycerol) for 3 hours at 4°C. Then this was mixed with 100 mg of dried IAM PC particles, and stirred for 1 hour at room temperature. The suspension of PGP-IAM was then dialyzed against dialysis buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM benzamidine) for 36 hours at 4°C (1.5 L for every 12 hours).

Preparation of liquid chromatographic column: The IAM PC particles with immobilized PGP were packed into a HR5/5 glass column (0.5 x 0.8 cm) after 3 times centrifugation at 350 x g, 3 min, at 4°C. Then the column was equilibrated with buffer B (50 mM Tris-HCl, pH 7.4) at room temperature for 3 hours.

Frontal chromatographic studies: The chromatographic system has been previously described (29) and was primarily based upon the PGP-IAM column connected on-line to a flow scintillation monitor (Radiometric FLO-ONE® Beta 500 TR instrument, Packard Instruments). All chromatographic experiments were conducted at room temperature using a flow rate of 0.5 ml/min.

The marker ligand, either [^{3}H]-vinblastine [[^{3}H]-VBL, 1.0 nM], [^{3}H]-verapamil [[^{3}H]-VER, 0.3 nM] or [^{3}H]cyclosporine A [[^{3}H]-CsA, 2.0 nM] were applied to the PGP-IAM column in sample volumes of 25-50 ml. The solutions containing the marker ligands were supplemented with a range concentrations of either cold VBL, VER, doxorubicin or CsA. Elution profiles were obtained showing front and plateau regions as illustrated for [^{3}H]VER in Figure 2. The observed elution volume data were used for calculation of ligand dissociation constants. The K_d values of VER and CsA were calculated by nonlinear regression, as illustrated for [^{3}H]-VER Fig. 3, using Prism (GraphPad Software) and a one-site binding (hyperbola), equation (2) below:

$$Y = Bmax \cdot X / (kd + X) \qquad (2)$$

In which: X is the concentration of VER or CsA; Y is equal to [verapamil] ($V - V_{min}$) or [CsA]($V - V_{min}$), where: V_{min} is the elution volume of VER or CsA under conditions where specific interactions are completely suppressed and V is the retention volume of VER or CsA at different concentrations (0.3 – 400 μM for VER and 2.5 - 100 nM for CsA).

Two series of runs were made to determine the K_d value for VBL and the K_i values for doxorubicin and CsA. One series was performed with different concentrations of cold VBL (3 nM – 100 nM) to displace [^{3}H]-VBL, and the other was performed with different concentrations of cold doxorubicin (5 μM – 70 μM) or CsA (10 nM – 250 nM) with [^{3}H]-VBL as the displaced ligand. The K_d value of VBL and the K_i values of doxorubicin and CsA were calculated using equations (3) and (4) below.

$$(Vmax - V)^{-1} = (1 + [VBL]K_{VBL}) \cdot (Vmin[P]K_{VBL})^{-1} + (1 + [VBL]K_{VBL})^2 \cdot (Vmin[P]K_{VBL}\,K_i)^{-1} \cdot [I]^{-1} \quad (3)$$

$$(V - V_{min})-1 = (V_{min}[P]K_{VBL}^{)-1} + (V_{min}[P])^{-1}[VBL] \quad (4)$$

Where: I represents doxorubicin or CsA; [P] the concentration of active receptor in the volume; V_{min}, the elution volume of VBL under conditions where the specific interaction is completely suppressed; V_{max} is the elution volume obtained with 1.0 nM [^{3}H]VBL.

Control Experiments: Membranes from the PGP-negative parental cell line, MDA436/LCC6 (30), were prepared and immobilized on an IAM support as described above. Using the procedure described above, the PGP-negative-IAM support was packed into a glass column (0.5 x 0.8 cm) and a second glass column (0.5 x 0.8 cm) was packed with untreated IAM support. The three columns, IAM support {negative control} PGP-negative-IAM {positive control}

and PGP-IAM {experimental} were separately connected on-line to a flow scintillation monitor and used in zonal chromatographic experiments. In these studies, a mobile phase composed of Tris-HCl [50 mM, pH 7.4] was constantly pumped through the column at a flow rate of 0.5 ml/min. A single 100 ml injection of the marker ligand, [^{3}H]VER [23.5 nM], was injected onto the column and the radioactive signal (CPM) was recorded every six seconds. The chromatographic data was evaluated in 0.5 minute intervals and smoothed using the Microsoft Excel program with a 5-point moving average.

Membrane Binding Assays: The binding assays were accomplished using a previously described method. Briefly, 50 µl of [^{3}H]VBL [3 nM – 100 nM with 2 % ethanol (v/v) was incubated with PGP-containing or PGP-negative membranes (150 µg in 50 µl) or bare IAM particles and 50 µl of cold VBL [12 µM] for two hours at room temperature. Bound and free drug were separated by rapid filtration through Whatman GF/C filters which had beed pre-soaked with 0.1% BAS inTris-HCl [50 mM, pH 7.4]. The filters were then washed with two portions of 5-ml ice-cold 20 mM Tris-HCl, 20 mM $MgCl_2$ buffer. The filters were dried, and retained radioactivity was quantitated by liquid scintillation counting. Specific binding was defined as the difference between total binding and nonspecific binding

Protein Assay: The amount of membrane and the immobilized membrane were determined by bicinchoninic acid (BAC) protein assay. The sample was diluted with NaOH [0.1 M]. A protein standard (0.3 – 37.5 µg in 50 µl) was prepared with Albumin standard (Pierce, Rockford, IL). The measurement procedure followed the instruction in Pierce BCA protein assay kit in which 20 ml of reagent A was mixed with 0.4 ml of reagent B. Aliquots [50 µl] of standards and samples were added in triplicate to a 96-well plate and 200 µl of

BCA reagent (A+B) was added in each well. The standards and samples were incubated at room temperature for three hours and the resulting absorbance at l = 570 nm was determined using a spectrophotometer. The amount of protein was calculated by using the Microsoft Excel program.

EXAMPLE 6:

Chromatographic studies with vinblastine and doxorubicin

The dissociation constants (K_d) of vinblastine (VBL) and doxorubicin were determined on the PGP-IAM stationary phase using displacement chromatography with [^{3}H]VBL as the marker ligand, Table 1 below.

TABLE 1

The k_d values calculated using frontal affinity chromatography on the immobilized PGP-IAM stationary phase

Drugs	k_d[a]	k_d
Vinblastine	23.5 ± 7.8nM	37.0 ± 10nM[b] 36.0 ± 5 nM[c]
Verapamil	54.2 ± 4.6μM	0.45 ± 0.05 mM[b]
Doxorubicin	15.0 ± 3.2μM[d]	31 ± 7.3 mM[b]
Cyclosporine A	62.5 ± 5.6 nMe	18 ± 3.6 nM[b] 97.9 ± 19.4 nM[d]

a. These values were measured in the present work

b. These values are from literature values.

c. It is from the literature value.

d. The values were obtained by displacing [^{3}H]vinblastine (see methods).

e. It was measured when 3 mM ATP was in the running buffer.

The calculated K_d of VBL was 23.5 ± 7.8 nM which is consistent with the previously reported values of 37.0 ± 10 nM (18) and 36 ± 5 nM. The K_d value of 15.0 ± 3.2 μM determined for doxorubicin was also consistent with the reported value of 31.0 ± 7.3 μM.

The chromatographic results were also consistent with the results obtained from binding assays using the same membranes utilized in the construction of the PGP-IAM stationary phase. In these studies, membrane extracts were prepared from the PGP-expressing cell line, MDA435/LCC6^{MDR1}, and the PGP-negative cell line, MDA435/LCC6 (30). VBL binding to the two membrane extracts and the IAM support was determined using a previously described rapid filtration method. No specific binding was observed with the PGP-negative cell membranes or the IAM particles, while a K_d value of 54.5 ± 40.8 nM was determined using the membranes from the PGP-expressing cell line. The calculated affinity was consistent with the previously published value, 37 ± 10 nM, obtained using the same experimental approach. In these experiments, it was necessary to add ethanol (2%, v/v) to the [^{3}H]VBL solution to prevent non-specific binding to the walls of the polypropylene tubes.

EXAMPLE 7:

Chromatographic studies with verapamil and vinblastine:

When verapamil (VER) was used as the displacer of the [^{3}H]VBL marker ligand, the calculated K_d value for VER was 54.2 ± 4.6 μM. This value was significantly higher than the previously reported values of 0.45 ± 0.05 μM, and 0.6 ± 0.18 μM. When the experimental conditions were reversed and [^{3}H]VER

was the marker ligand and VBL the displacer, no displacement of [^{3}H]VER was observed when 50 nM and 100 nM concentrations of VBL were added to the mobile phase, Table 2 below.

TABLE 2

Retention volumes of [^{3}H]vinblastine and [^{3}H]cyclosporine A obtained when (1) no ATP was present in the running buffer, (2) 3 mM ATP was added in the running buffer, (3) 50 nM cold vinblastine supplemented in the sample (no ATP in the buffer), and (4) 100 nM cold vinblastine was in the sample (no ATP in the buffer).

Drugs	Retention volume (ml) at			
	No ATP	3 mM ATP	50 nM vinblastine (no ATP)	100 nM vinblastine (noATP)
[^{3}H]Vinblastine	32.1	8.4	11.0	9.5
[3H]verapamil	34.2	5.9	34.1	34.0
[^{3}H]Cyclosporine A	7.8	17.5	15.7(15.4)[a]	18.8

a.15.7 ml was measured at the condition of no ATP was present in the running buffer, and 15.4 ml was obtained when 3 mM ATP was in the running bufer.

The specificity of the chromatographic interactions of VER with the immobilized PGP were investigated through the independent immobilization of membrane extracts from the PGP-expressing cell line and the PGP-negative cell line on the IAM-support. Zonal chromatographic studies were conducted with columns containing either the PGP-IAM, PGP-negative-IAM or IAM support. When a 100 ml sample of [^{3}H]VER was injected onto the columns containing

either the PGP-negative-IAM support or the IAM support itself, the retention volumes were the same, Figure 4, curves 1 and 2. This indicates that there was no specific interactions with the immobilized membrane extracts obtained from the PGP-negative cells relative to the non-specific interaction that occur between [^{3}H]VER and the IAM support. On the column containing the PGP-IAM support, the retention volume of [^{3}H]VER was > 20 ml, Figure 4, curve 3. This indicates that specific binding interactions occurred between the [^{3}H]VER and the immobilized membrane extracts obtained from the PGP-expressing cells.

EXAMPLE 8:

Chromatographic studies with cyclosporin A and vinblastine:

When cyclosporin A (CsA) was used as the displacer of the [^{3}H]VBL marker ligand, the calculated K_d value for CsA was 97.9 ± 19.4 nM as compared to the previously reported value of 18.0 ± 3.6 nM, Table 1. When [^{3}H]CsA was used as the marker ligand and migrated alone through the PGP-IAM, the retention volume was 7.8 ml, Table 2, and no specific retention was observed, Fig. 5A. The addition of 50 nM VBL to the running buffer increased the retention volume of [^{3}H]CsA to 15.7 ml, Table 2, and produced the expected frontal chromatogram, Fig. 5B. When the VBL concentration was increased to 100 nM, the observed retention of the frontal chromatogram increased to 18.8 ml, Fig. 5D,Table 2.

EXAMPLE 9:

Effect of ATP on the chromatographic properties of the PGP-IAM:

The addition of 3 mM ATP to the running buffer resulted in changes in the retentions of CsA, VBL and VER. In the case of CsA, the addition of ATP increased the retention volume from 7.8 ml to 17.5 ml, Table 2, and the observed chromatogram changed from non-specific elution, Fig. 5.A, to a frontal

chromatogram displaying specific retention to the immobilized PGP-IAM, Figure 4. With 3mM ATP in the running buffer, [^{3}H]CsA was displaced from PGP by the addiiton of unlabeled-CsA. The results fom the CsA displacement studies were used to calculate a K_d value of 62.5 nM for CsA binding to the immobilized PGP.

When VBL was the marker ligand, the addition of 3 mM ATP decreased the retention volume from 32.1 ml to 8.4 ml, Table 2. The presence of ATP in the running buffer also changed the observed chromatograms from a frontal curve demonstrating specific retention, Figure 6A, to a non-specific curve, Figure 6B. A similar effect was observed for VER as the addition of 3 mM ATP to the running buffer reduced the elution volume from 34.2 ml to 5.9 ml, Table 2, with a resulting loss in specific retention, as demonstrated by the shape of the frontal curve (data not shown).

CONCLUSIONS

The synthesis and characterization of a novel liquid chromatographic stationary phase containing immobilized P-glycoprotein (PGP-IAM) is described *supra*. The stationary phase was prepared using solubilized membranes from PGP-expressing cells. The donor cells were MDA435/LCC6^{MDR1}, a cell line obtained by retroviral transduction of MDR1 cDNA (coding for PGP) into MDA435/LCC6 human breast cancer cells. In this study, a second liquid chromatographic stationary phase was prepared through the immobilization of membrane extracts from the PGP-negative MDA435/LCC6 parental cell line. A comparison of the chromatographic retention of verapamil, a known PGP substrate, on the native chromatographic support and the PGP-positive and PGP-negative supports, Figure 5 demonstrated that, for PGP substrates, the observed

chromatographic retentions were a function of specific interactions between the substrate and the immobilized PGP.

The relationship between chromatographic retention on the PGP-IAM stationary phase and PGP binding affinity was also illustrated by the comparison of the substrate affinities calculated from the chromatographic results obtained using the PGP-IAM column with the results from classical filtration binding assays, Table 1. The initial studies in this series were conducted using [^{3}H]-vinblastine ([^{3}H]VBL) as the marker ligand and Tris buffer [50 mM, pH 7.4] as the running buffer. Under these conditions cyclosporin A (CsA) displaced [^{3}H]VBL producing a calculated K_d value of 97.9 nM, Table 1, which is consistent with the results of previous studies based on filtration binding assays.

The displacement of [^{3}H]VBL by CsA indicated that CsA specifically and competitively bound to immobilized PGP. However, when [^{3}H]CsA was used as the marker ligand, frontal chromatography with [^{3}H]CsA alone in the running buffer produced a low retention volume, 7.8 ml (Table 2) and no detectable specific retention, see Figure 5A. This indicated that under the experimental conditions, [^{3}H]CsA did not specifically bind to immobilized PGP.

The contradiction between the data obtained with [^{3}H]VBL as the marker versus the data obtained with [^{3}H]CsA alone in the running buffer was eliminated when VBL was added to the running buffer. The addition of 50 nM VBL to the running buffer produced a classical frontal chromatogram for [^{3}H]CsA (Figure 5B) and increased the retention volume to 15.7 ml, Table 2. When the VBL concentration was increased to 100 nM, the retention volume also increased to 18.8 ml, Table 2, Figure 5D.

The results from the studies with [^{3}H]VBL and [^{3}H]CsA as the marker ligands indicate that the addition of VBL to the running buffer produces a co-

operative allosteric interaction in the binding process between [^{3}H]CsA and the immobilized PGP. This suggests that the immobilized PGP has retained its conformational mobility and that the binding of VBL to PGP opened up the site at which CsA binds.

The data also indicates that once the VBL-induced conformational change has occurred, CsA can bind to PGP and displace VBL through competitive and/or anti-cooperative allosteric interactions. The addition of CsA to the running buffer did not change the shape of the [^{3}H]VBL frontal chromatograms demonstrating that the displacement was competitive in nature. One explanation for these results is that the VBL induced CsA binding site is contiguous with or part of the VBL site. CsA binding to the induced site on PGP does not directly compete with VBL for the same site, but inhibits VBL binding through steric interactions. Korzekawa, et al. have proposed a similar model for enzymatic inhibition as well as activation in some isoforms of cytochrome P450. In this model, the simultaneous but independent binding of two different substrates in the active site of the enzyme results in steric interactions that produce the displacement {inhibition} or re-orientation {activation} of one of the substrates.

In these studies, the addition of increasing concentrations of VER to the running buffer reduced the retention volumes of [^{3}H]VBL without changing the shapes of the frontal chromatograms. This indicates that VER competitively displaced VBL from its binding to PGP, although the calculated K_d value was significantly higher than previously reported values, Table 1. However, VBL was unable to displace [^{3}H]VER from the immobilized PGP. These results suggest that VER binds to two or more distinct sites on the PGP molecule including the site at which VBL binds. Furthermore, the site that is common to VBL and VER is not the primary VER binding site, i.e., the site for which VER

has the highest affinity. Thus, the Kd value calculated from the frontal chromatographic studies, Table 1, appears to be the sum of VER affinity to the different VER binding sites. The experimental conditions used in this study could not determine if the VER and VBL sites are allosterically linked. Further studies will be effected to select specific markers for the high and low affinity VER binding sites.

The existence of multiple binding sites on the PGP molecule has been suggested by the results of several previous studies. Using classical filtration binding assays, Ferry et al. Obtained evidence of non-overlapping binding sites for Vinca alkaloids and dihydropyridine substrates, and perhaps also for Vinca alkaloids and doxorubicin. Also, distinct sites for steroids and Vinca alkaloids, steroids and VER, VER and dihydropyridines, and between different steroids, were supported by the results of studies using an ATPase activation endpoint. Moreover, separate binding sites have been suggested for VER and anthracyclines, VER and colchicine, and cyclosporins and dihydropyridines.

PGP is a member of the ATP-binding cassette superfamily and ATPase activity plays a role in substrate transport. Thus, it should be expected that the addition of ATP to the running buffer would change the chromatographic properties of the immobilized PGP chromatographic system. In this study, the addition of 3 mM ATP to the running buffer increased the retention volume of [^{3}H]CsA from 7.8 ml to 17.5 ml (Table 2), produced a classical frontal chromatogram for [^{3}H]CsA (Figure 5C) and permitted the calculation of an a k_d value of 62.5 nM, Table 1. These results indicate that the addition of ATP to the running buffer produced a cooperative allosteric interaction that increased the binding affinity of PGP for CsA.

The presence of ATP in the running buffer produced the opposite effect on the retention volumes of [^{3}H]VBL and [^{3}H]VER. When [^{3}H]VBL was the marker ligand, the addition of 3 mM ATP reduced the observed retention from 32.1 ml to 8.4 ml, Table 2. The reduction in [^{3}H]VBL retention volume was accompanied by an apparent loss of specific retention, Figure 4, suggesting an anti-cooperative allosteric interaction. This possibility is supported by the results of VBL displacement experiments. The addition of unlabeled VBL to the running buffer, in the absence of 3 mM ATP also decreased the retention volume of [^{3}H]VBL, Table 2. However, the effect of VBL on [^{3}H]VBL retention differs from the effect observed with ATP as specific frontal chromatographic curves were observed after the addition of 50 nM and 100 nM VBL {data not shown}. This indicates that the decreased retention was due to competitive displacement of [^{3}H]VBL by VBL. The ATP induced reduction in the retention volume for [^{3}H]VER, 34.2 ml to 5.9 ml, coupled with the loss of specific retention, also indicate that the primary site of VER binding to the PGP molecule was affected by an anti-cooperative allosteric interaction.

These results support the observations from the VBL-CsA binding interaction studies that the immobilized PGP has retained it conformational mobility. Thus, the addition of ATP to the running buffer produced a conformational change in the immobilized PGP molecule that opened up the site at which CsA binds. The same conformational change that increased the binding affinity for CsA also altered the site at which VBL binds, decreasing the affinity of PGP for VBL. The effect of VBL on CsA binding affinity and the effect of ATP on the binding affinities of VBL and CsA indicates that separate, but closely linked, binding sites for CsA and VBL exist on the PGP molecule.

The observation of ATP-induced conformational shifts in the immobilized PGP molecule is consistent with results from previous studies. An infrared spectroscopy study has shown that PGP changes conformation a first time when ATP is added, and then again when a substrate is added in the presence of ATP. The changes in affinity for VBL and CsA that we observed following the addition of ATP most probably reflects the second of the above conformational changes, or the situation that immediately follows substrate binding and ATPase activation.

To our knowledge, the results reported herein, and specifically the dramatic effect of VBL on CsA binding to PGP, are the first to strongly suggest distinct and allosterically connected sites for Vinca alkaloids (VBL) and cyclosporins. The results of previous studies of dissociation rates were consistent with overlapping binding sites for CsA and VBL.

The mechanism by which PGP causes the efflux of several substrates has been discussed for many years and remains incompletely defined. In the most widely accepted model, PGP is proposed to work as an "hydrophobic vacuum cleaner", binding its substrates from the inner leaflet of cell membrane and transporting them to the extracellular space, or perhaps "flipping" them to the outer leaflet. Both transport mechanisms assume a conformationally mobile molecule capable of responding to ATPase activity and substrate/inhibitor binding.

The immobilized PGP liquid chromatographic stationary phase described herein appears to reproduce PGP substrate binding as determined by classical filtration binding assays. Observed binding in this system is PGP-specific and highly sensitive to conformational changes caused by PGP interactions with substrates and ATP, reflecting changes occurring in the functional cycle of

PGPGP Thus, PGP-affinity chromatography represents a promising tool for a quick and reproducible evaluation of potential PGP substrates and/or inhibitors and provides a useful probe of the transport mechanism.

WHAT IS CLAIMED IS:

1. A method for identifying, isolating and/or characterizing ligands that interact with p-glycoprotein comprising:

(i) immobilizing p-glycoprotein on an artificial membrane support which is on-line with a liquid chromatography system; and

(ii) continuously contacting said immobilized P-glycoprotein with a liquid chromatographic system that contains one or more ligands that are to be identified, isolated and/or characterized based on their specific interaction with said immobilized P-glycoprotein.

2. The method of Claim 1 which further comprises eluting ligands that bind to P-glycoprotein on said artificial membrane support.

3. The method of Claim 1, which further comprises evaluating the elution profile of said ligands.

4. The method of Claim 1, wherein an identified ligand is evaluated to determine its effect on breast cancer cells *in vitro*.

5. The method of Claim 1, wherein an identified ligand is evaluated in an animal xenograft model of human breast cancer.

6. The method of Claim 1, wherein the immobilized PGP is complexed with a compound that specifically binds PGP and the method is used

to continuously identify ligands that specifically bind PGP by a displacement competition binding assay.

7. The method of Claim 1, wherein said identified ligands are further characterized by passing an eluate containing said ligand through another testing device.

8. The method of Claim 7, wherein said device is a mass spectrometer.

9. The method of Claim 1, which is used to identify compounds for treating MDR1/PGP resistant tumors.

10. The method of Claim 1 which comprises more than one chromatographic screen using columns comprising different P-glycoprotein-containing compounds or derivatives.

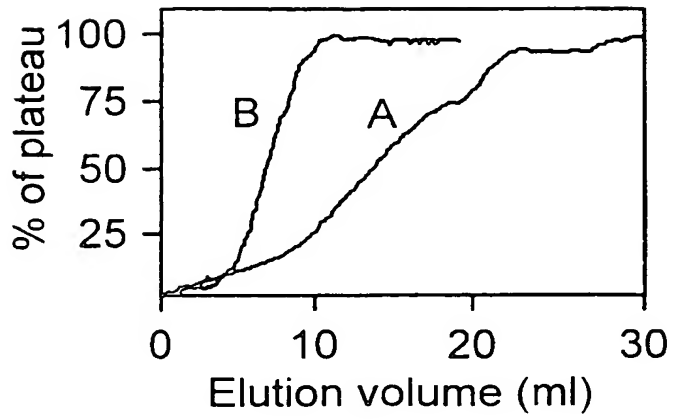
% of plateau
100
75
50
25
B
A
0
10
20
30
Elution volume (ml)

FIG. 1

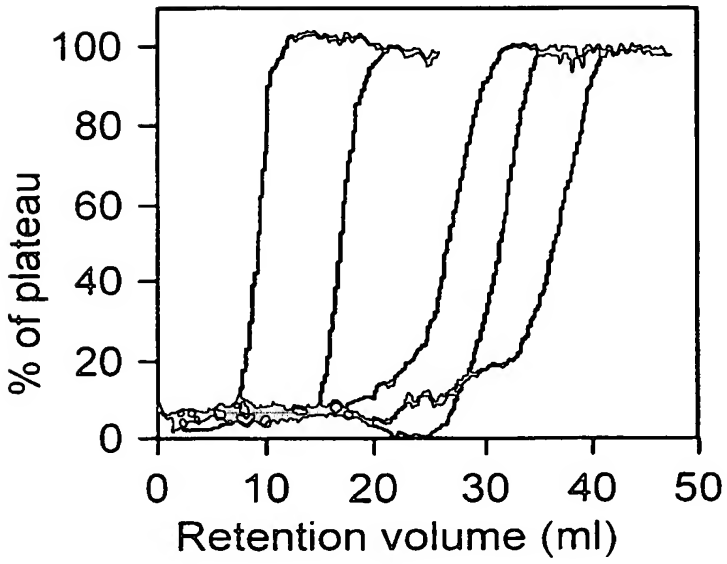
% of plateau
100
80
60
40
20
0
0
10
20
30
40
50
Retention volume (ml)

FIG. 2

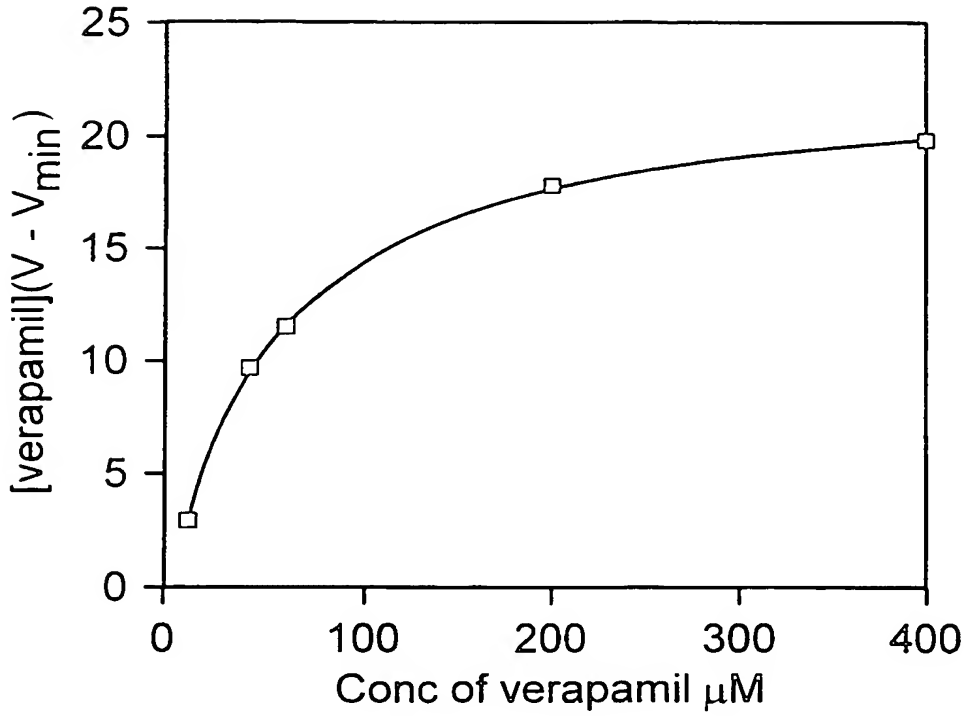
25
20
15
10
5
0
[verapamil](V - Vmin)
0
100
200
300
400
Conc of verapamil μM

FIG. 3

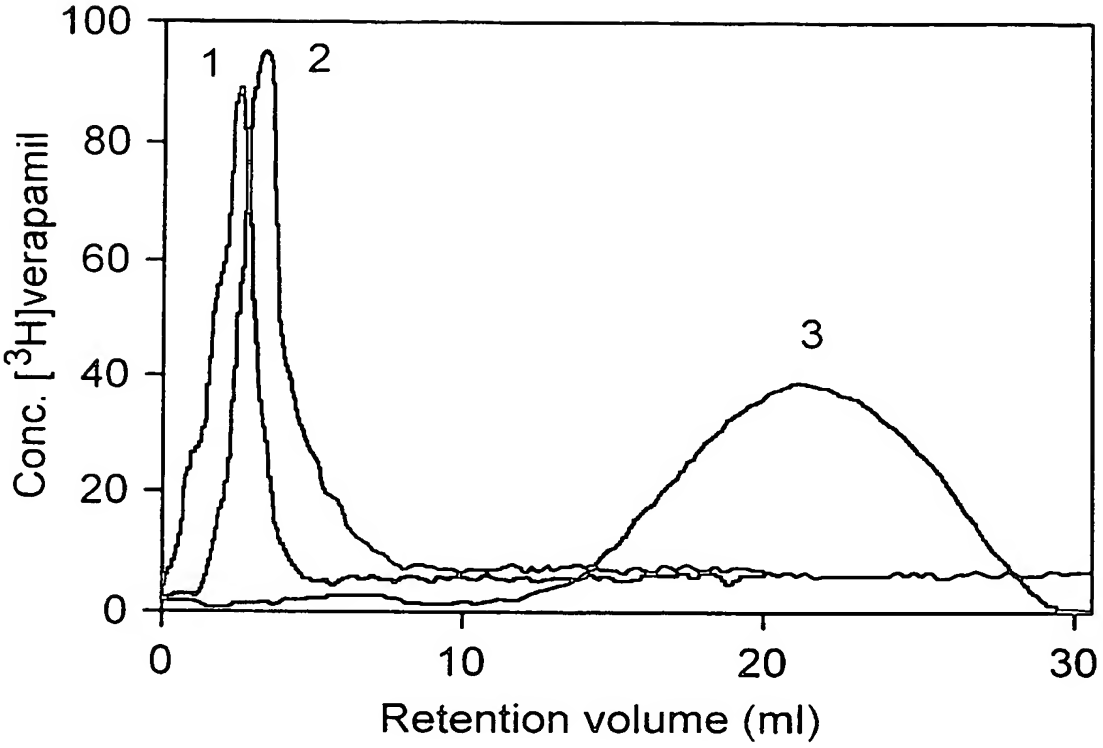
100
80
60
40
20
0
Conc. [3H]verapamil
1
2
3
0
10
20
30
Retention volume (ml)

FIG. 4

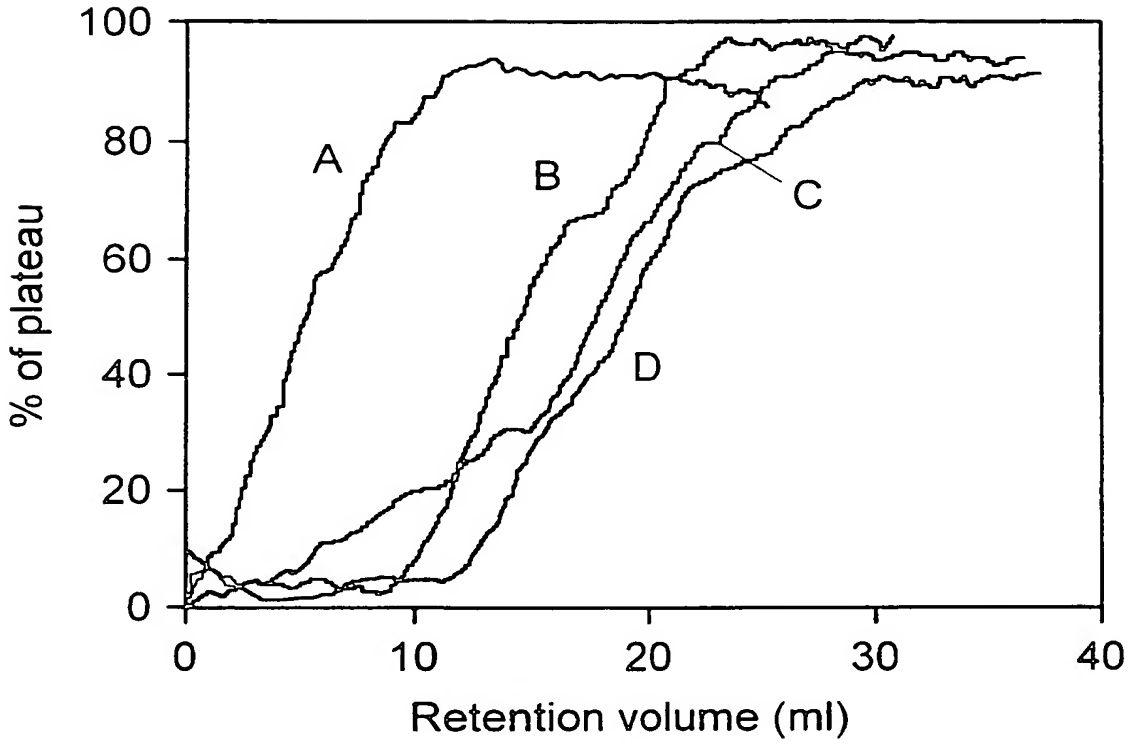
100
80
60
40
20
0
% of plateau
A
B
C
D
0
10
20
30
40
Retention volume (ml)

FIG. 5

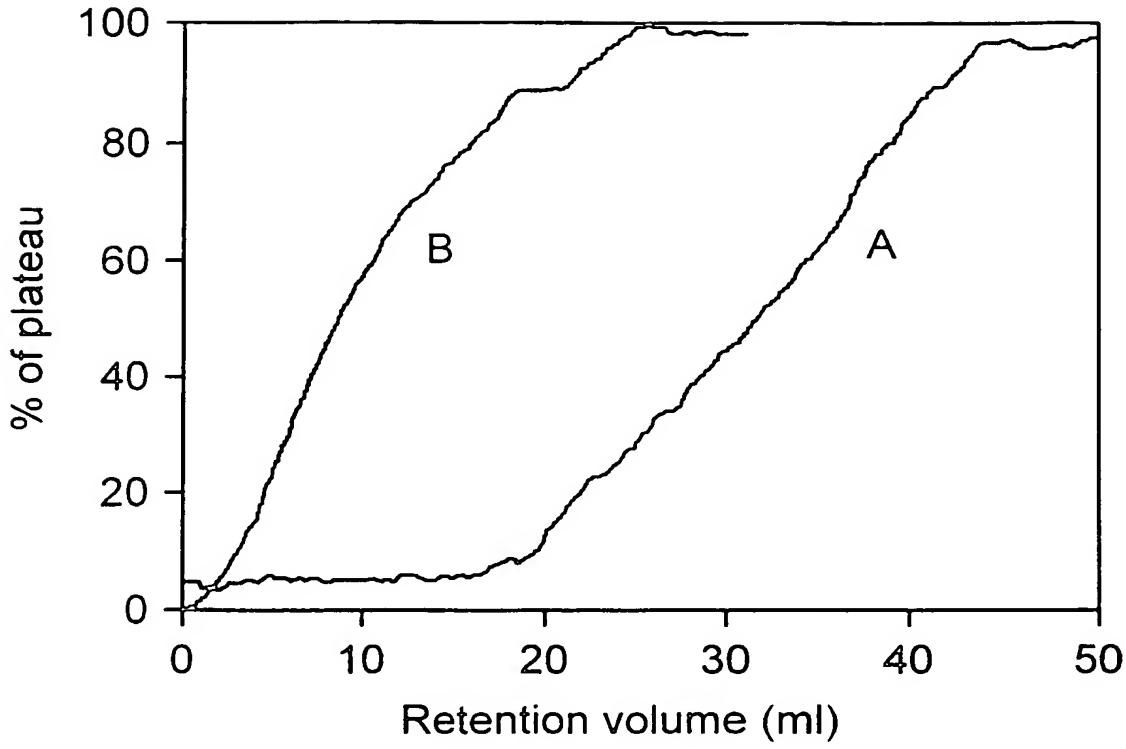
100
80
60
40
20
0
% of plateau
B
A
0
10
20
30
40
50
Retention volume (ml)

FIG. 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/15820

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C07K 14/00, 16/00; C12P 7/00, 29/00; G01N 33/537, 33/574, 33/577

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : Please See Extra Sheet.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE,EMBASE,SCISEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 4,837,306 A (LING et al.) 06 June 1989 (06.06.89), see entire document.	1-3,10
X,P	ZHANG et al. "Development of an Immobilized P-glycoprotein Stationary Phase for on-line Liquid Chromatographic determination of Binding Affinities," Journal of Chromatography. B, Biomedical Sciences and Applications. February 2000. Vol. 739. No. 1. pages 33-37, see entire document.	1-10
Y	GIANNI et al. "Human Pharmacokinetic Characterization and In-vitro study of the Interaction between Doxorubicin and Paclitaxel in patient with Breast Cancer," Journal of Clinical Oncology. May 1997. Vol. 15. No. 5. pages 1906-1915, see entire document.	4-9

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

22 AUGUST 2000

Date of mailing of the international search report

03 OCT 2000

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

GAILENE R. GABEL

Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/15820

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y,P	SPARREBOOM et al. Clinical Pharmacokinetics of Doxorubicin in combination with GF 120918, a Potent Inhibitor of MDR1-P-glycoprotein, Anti-cancer Drugs. September 1999. Vol. 10. No. 8. pages 719-728. see entire document.	4-9
Y	KUBOTA et al. Pirarubicin might partly circumvent the P-glycoprotein-mediated Drug Resistance of Human Breast Cancer Tissues. Anticancer Research March-April 1998. Vol. 18. No. 2A. pages 967-972. see entire document.	4-9

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/15820

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

530/350, 355, 387.7, 388.8, 388.85, 389.7, 395; 435/7.1, 7.2, 7.23, 7.92, 7.93; 436/538, 541, 64, 161, 162, 163, 167, 173, 174, 813, 824

B. FIELDS SEARCHED
Minimum documentation searched
Classification System: U.S.

530/350, 355, 387.7, 388.8, 388.85, 389.7, 395; 435/7.1, 7.2, 7.23, 7.92, 7.93; 436/538, 541, 64, 161, 162, 163, 167, 173, 174, 813, 824

DO/ EO WORKSHEET

U.S. Appl. No. 10/009344 International Appl. No. US0015820

Application filed by : ☐ 20 months ☐ 30 months

WIPO PUBLICATION INFORMATION :

Publication No.: WO 01/75179 Publication Language : ☐ English ☐ Japanese ☐ German ☐ French ☐ Other : _____ Screening Done by :

Publication Date : 4 Oct 01 Not Published : ☐ U.S. only designated ☐ EP request

INTERNATIONAL APPLICATION PAPERS IN THE APPLICATION FILE :

- ☑ International Application *(RECORD COPY)*
- ☐ Article 19 Amendments
- ☑ PCT/IB/331
- ☑ PCT/IPEA/409 IPER (PCT/IPEA/416 on front)
- ☐ Annexes to 409
- ☐ Priority Document (s) No. _____
- ☐ International Appl. on Double Sided Paper *(COPIES MADE)*
- ☐ Request form PCT/RO/101
- ☑ PCT/ISA/210 - Search Report
- ☐ Search Report References
- ☐ Other : _____

RECEIPTS FROM THE APPLICANT *(other than checked above) :*

- ☑ Basic National Fee (*or authorization to charge*)
- ☑ Description
- ☑ Claims 10/1
- ☑ Words in the Drawing Figure(s) 5
- ☐ Article 19 Amendments
  - ☐ english transl. of annexes NOT present
  - ☐ entered ☐ not entered :
    - ☐ not a page for page substitution
    - ☐ other : _____
- ☐ Annexes to 409
  - ☐ english transl. of annexes NOT present
  - ☐ entered ☐ not entered :
    - ☐ not a page for page substitution
    - ☐ other : _____
- ☐ Preliminary Amendment(s) Filed on : 1. _____ 2. _____ 3. _____
- ☐ Information Disclosure Statement(s) Filed on : 1. _____ 2. _____ 3. _____
- ☐ Assignment Document
- ☐ Power of Attorney/ Change of Address
- ☐ Substitute Specification Filed on : 1. _____ 2. _____
- ☐ Verified Small Status Statement (executed)
- ☐ Oath/ Declaration (executed)
  - ☐ surcharge was paid at the time of filing
- ☐ DNA Diskette
- ☐ Other : 1. _____ 2. _____

NOTES : ☐ I.A. used as Specification ☐ Other :

35 U.S.C. 371 - Receipt of Request (PTO-1390)	10 Dec 01
Date Acceptable Oath/ Declaration Received	
Date of Completion of requirements under 35 U.S.C. 371	
102(e) Date	
Date of Completion of DO/ EO 906 - Notification of Missing 102(e) Requirements	
Date of Completion of DO/ EO 907 - Notification of Acceptance for 102(e) Date	
Date of Completion of DO/ EO 911 - Application Accepted Under 35 U.S.C. 111	
Date of Completion of DO/ EO 905 - Notification of Missing Requirements	07 Feb 02
Date of Completion of DO/ EO 916 - Notification of Defective Response	
Date of Completion of DO/ EO 903 - Notification of Acceptance	

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference 010091-144	**FOR FURTHER ACTION**	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/US00/15820	International filing date *(day/month/year)* 09 June 2000 (09.06.2000)	Priority date *(day/month/year)* 09 June 1999 (09.06.1999)
International Patent Classification (IPC) or national classification and IPC IPC(7): C07K 14/00, 16/00; C12P 7/00, 29/00; G01N 33/537, 55/574, 33/577 and US Cl.: 530/350, 355, 387.7, 388.8, 388.85, 389.7,395; 435/7.1, 7.2, 7.23, 7.92, 7.93; 436/538, 541, 64, 161, 162, 163, 167, 173, 174, 813, 824		
Applicant GEORGETOWN UNIVERSITY		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 4 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 0 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of report with regard to novelty, inventive step and industrial applicability
   - IV ☒ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
09 January 2001 (09.01.2001)	20 October 2001 (20.10.2001)
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703)305-3230	Authorized officer Gailene R. Gabel Telephone No. (703) 308-0196

Form PCT/IPEA/409 (cover sheet)(July 1998)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US00/15820

I. Basis of the report

1. With regard to the **elements** of the international application:*

☒ the international application as originally filed.

☒ the description:
pages 1-31 as originally filed
pages NONE, filed with the demand
pages NONE, filed with the letter of _____.

☒ the claims:
pages 32 and 33, as originally filed
pages NONE, as amended (together with any statement) under Article 19
pages NONE, filed with the demand
pages NONE, filed with the letter of _____.

☒ the drawings:
pages 1-5, as originally filed
pages NONE, filed with the demand
pages NONE, filed with the letter of _____.

☐ the sequence listing part of the description:
pages NONE, as originally filed
pages NONE, filed with the demand
pages NONE, filed with the letter of _____.

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language _____ which is:

☐ the language of a translation furnished for the purposes of international search (under Rule23.1(b)).

☐ the language of publication of the international application (under Rule 48.3(b)).

☐ the language of the translation furnished for the purposes of international preliminary examination(under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

☐ contained in the international application in printed form.

☐ filed together with the international application in computer readable form.

☐ furnished subsequently to this Authority in written form.

☐ furnished subsequently to this Authority in computer readable form.

☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. ☐ The amendments have resulted in the cancellation of:

☐ the description, pages NONE

☐ the claims, Nos. NONE

☐ the drawings, sheets/~~fig~~ NONE

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).**

** Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).*
*** Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.*

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US00/15820

IV. Lack of unity of invention

1. In response to the invitation to restrict or pay additional fees the applicant has:
   - ☐ restricted the claims.
   - ☐ paid additional fees.
   - ☐ paid additional fees under protest.
   - ☐ neither restricted nor paid additional fees.

2. ☐ This Authority found that the requirement of unity of invention is not complied with and chose, according to Rule 68.1, not to invite the applicant to restrict or pay additional fees.

3. This Authority considers that the requirement of unity of invention is accordance with Rules 13.1, 13.2 and 13.3 is
   - ☒ complied with.
   - ☐ not complied with for the following reasons:

4. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this report:
   - ☐ all parts.
   - ☐ the parts relating to claims Nos. ____

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US00/15820

V. Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. STATEMENT

Novelty (N)	Claims	1-10	YES
	Claims	NONE	NO
Inventive Step (IS)	Claims	NONE	YES
	Claims	1-10	NO
Industrial Applicability (IA)	Claims	1-10	YES
	Claims	NONE	NO

2. CITATIONS AND EXPLANATIONS

Claims 1-10 lack an inventive step under PCT Article 33(3) as being obvious over Ling et al. (US 4,837,306) in view of Kubota et al. (Anticancer Research, 1998) and Gianni et al. (Journal of Clinical Oncology, 1997).

Ling et al. disclose that P-glycoprotein surface antigen correlates well with multidrug resistance and therefore raises the possibility that P-glycoprotein may serve as a diagnostic marker for the detection of multidrug-resistant cells in cancer patients (see Abstract). Ling et al. specifically disclose immobilizing P-glycoprotein on artificial membrane support (nitrocellulose filters) to detect ligands (antibodies) under study for identification, isolation or characterization (see column 7, specially lines 28-32).

Ling et al. differ in failing to incorporate P-glycoprotein into a liquid chromatography system for use in order to identify the activity of certain ligands with P-glycoprotein.

Kubota et al. teach assessing the sensitivity and expression of P glycoprotein with ligands such as anthracyclines (Doxorubin and Tetrahydropyranyl Adriamycin (see page 969).

Gianni et al. teach assessing the pharmacokinetic activity of doxorubin and other ligands using high pressure liquid chromatography (see Abstract).

One of ordinary skill in the art at the time of the instant invention would have reasonable expectation of success in incorporating the teaching of Gianni in using HPLC to assess pharmacokinetic acitivity of ligands that interact with PGP such as anthracyclines as taught by Kubota, into the method of Ling which specifically disclosed immobilizing PGP onto a membrane support to detect and assess chatacterization of ligands that interact with PGP because incorporation of bioaffinity matrices such as suggested by Ling into chromatographic systems is conventional and well within ordinary skill in the art.

Claims 1-10 have industrial applicability as defined by PCT Article 33(4) in the field of combinatorial chemistry and drug screening assays.

---------- NEW CITATIONS ----------